

1 **Review**

2 **Formation of clustered DNA Damage after High-LET Irradiation: A review**

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1 **Formation of clustered DNA Damage after High-LET Irradiation: A review**

3 **Clustered DNA Damage/High-LET Radiations**

4 Radiation can cause as well as cure cancer. The risk of developing radiation-induced cancer
5 has traditionally been estimated from cancer incidence among survivors of the atomic bombs in
6 Hiroshima and Nagasaki ¹⁾. These data provide the best estimate of human cancer risk over the
7 dose range for low linear energy transfer (LET) radiations, such as X- or γ -rays. The situation of
8 estimating the real biological effects becomes even more difficult in the case of high LET
9 particles encountered in space or as the result of domestic exposure to α -particles from radon gas
10 emitters or other radioactive emitters like uranium-238.

11 Complex DNA damage, i.e., the signature of high-LET radiations comprises by closely
12 spaced DNA lesions forming a cluster of DNA damage. The two basic groups of complex DNA
13 damage are double strand breaks (DSBs) and non-DSB oxidative clustered DNA lesions
14 (OCDL). Theoretical analysis and experimental evidence suggest there is increased complexity
15 and severity of complex DNA damage with increasing LET (linear energy transfer) and a high
16 mutagenic or carcinogenic potential. Data available on the formation of clustered DNA damage
17 (DSBs and OCDL) by high-LET radiations are often controversial suggesting a variable response
18 to dose and type of radiation. The chemical nature and cellular repair mechanisms of complex
19 DNA damage have been much less characterized than those of isolated DNA lesions like an
20 oxidized base or a single strand break especially in the case of high-LET radiation. This review
21 will focus on the induction of clustered DNA damage by high-LET radiations presenting the
22 earlier and recent relative data.

1 INTRODUCTION TO THE IDEA OF CLUSTERED DNA DAMAGE

2 The 'idea' of clustered DNA damage was first introduced by Ward as *locally multiple damaged*
3 *sites* (LMDS), i.e., several closely spaced damages within a short DNA segment that could be
4 produced by ionizing radiation ^{2,3)}. Ward introduced the idea of clustered DNA lesions to
5 account for the increased lethality induced by ionizing radiation, which cannot be fully explained
6 by the amount of double strand breaks formed, although the specific lesions, if unrepaired or
7 misrepaired, can lead to a lethal event.

8 Random energy deposition by ionizing radiation induces a wide variety of DNA lesions
9 [single (SSBs) and double strand breaks (DSBs), oxidized bases and apurinic-aprimidinic
10 (abasic, AP) sites] ⁴⁾. Ionizing radiation induces damage in DNA by direct ionization and through
11 generation of hydroxyl radicals that attack DNA and induce some or all of the above lesions
12 (indirectly). In addition to the prompt breaks induced by radiation, some post-irradiation ones
13 can be also formed as the result of the attempted repair of some sugar and base residues induced
14 directly which can later be converted to SSBs or DSBs ⁵⁾. Two or more DNA lesions of the same
15 or different nature may be produced in close proximity to each other on opposite DNA strands
16 (bistranded lesions), generally within one-two helical turns of the DNA molecule. Theoretical ⁶⁾
17 and experimental studies ⁷⁾ support the induction of these clustered DNA lesions in the cellular
18 environment or high scavenger conditions by a single radiation track and not by multiple track
19 events. These various closely spaced bistranded types of DNA damage are called clustered DNA
20 damages and can include SSBs of varying complexity, oxidized base lesions (oxypurines and
21 oxypyrimidines: oxybases) and regular as well as oxidized AP sites (Fig. 1). In general clustered
22 DNA damage can be separated to two major groups: DSBs and non-DSB oxidative clustered
23 DNA lesions, OCDL ⁸⁾. Even at doses as low as ~1 Gy (100 rad) ionizing radiation is capable of

1 inducing all of the above types of DNA damage in the form of isolated lesions as well as in the
2 form of clustered ones (1-10 bp apart)^{9,10)}. Except ionizing radiation, other radiomimetic drugs
3 like bleomycin or neocarzinostatin have been shown to induce DNA damage in a form of a
4 cluster explaining in part the high toxicity of these drugs^{11,12)}. It has been shown that clustered
5 lesions constitute 50-80% of the total complex DNA damage¹³⁾. Simultaneous processing of the
6 lesions located on opposite strands may generate additional DSBs in addition to the ones directly
7 induced by ionizing radiation and enhance genomic instability^{14,15)}. In fact, *E. coli* and rodent
8 cells do generate *de novo* DSBs that could result from processing of bistranded clustered DNA
9 lesions^{16,19)}. There are very limited data on the possible accumulation of OCDLs in human cells
10 or tissues and in mammalian/human tissues exposed to ionizing radiation²⁰⁾. Gollapalle *et al.*⁸⁾
11 showed for the first time that endogenous OCDL can be detected in non-irradiated mice tissues
12 at a steady state of ~0.3-0.8 clusters/Mbp and that exposure of these tissues to ionizing radiation
13 (X-rays) leads to an accumulation of DNA clusters detected 20 weeks after irradiation.
14 Measurement of endogenous OCDL in the human breast cancer line MCF-7 revealed elevated
15 levels compared with the non-malignant MCF-10A. These cluster frequencies are higher than
16 the ones detected for DNA isolated from human skin primary cell cultures (20-40 clusters/Gbp or
17 0.02-0.04 clusters/Mbp)²¹⁾. These yields discrepancies may be due to different cell types or
18 different isolation and enzyme-treatment methods for DNA analysis.

19 Cytotoxic effects of ionizing radiation are thought to result principally from incompletely or
20 incorrectly repaired DNA lesions²²⁾. While isolated damages are generally repaired efficiently,
21 clustered DNA lesions have been suggested to be more difficult to repair, and in general are
22 considered as DNA damages that are repair-resistant or non-repairable with a high mutagenic
23 potential and, therefore, considered as highly significant biological endpoints^{2,9)}. A significant

1 number of studies suggest that the mutation frequency increases the closer the spacing of the
2 clustered DNA lesions ^{23,25)}. DNA clusters could be resistant to processing by glycosylases or
3 endonucleases, as shown for synthetic oligonucleotides containing clusters of specific
4 composition and configuration ^{26,32)}. The majority of references on repair of clusters reflect
5 retarded enzymatic activity. Such repair-resistant clusters could persist for a substantial time
6 after irradiation ^{33,34)}.

7

8 **PRINCIPLES OF MEASUREMENT OF CLUSTERED DNA LESIONS**

9 Here we aim to give the principles of the approaches used from different laboratories for
10 the detection of bistranded clustered DNA lesions as well as the general description of the way
11 that they were evolved and fine-tuned for an efficient detection of closely spaced opposed
12 oxidative DNA lesions in DNA isolated from human or mammalian cells, genomic DNA like T7
13 or λ -DNA or supercoiled DNA ^{35,36)}.

14 A unique approach for quantifying these types of bistranded damage in human cells using
15 DNA repair enzymes isolated from *E. coli* was initially developed by Sutherland *et al.* ¹⁰⁾. The
16 idea relies on the fact that repair enzymes participating in base excision repair (BER) like DNA
17 glycosylases and AP endonucleases will function also *in vitro*, i.e., on isolated DNA carrying
18 clustered lesions. Once they detect the lesion (Fig. 1 and Table 1) in each cluster, they will excise
19 it and cleave the DNA strand by their intrinsic lyase activity (DNA glycosylases) or cleave
20 directly in the case of an AP endonuclease and create a SSB in each strand, i.e., a DSB in the
21 case of a cluster (Fig. 1). As shown in Table 1, there is an overlapping specificity of Fpg,
22 EndoIII and EndoIV towards abasic sites as well as between Fpg and EndoIII towards some
23 types of oxidized bases. The additional DSBs induced by the repair enzymes can be measured

1 using constant field or pulsed field gel electrophoresis (PFGE) following number average length
2 analysis (NALA) according to the size of extracted DNA and are considered equal to the clusters
3 detected by the enzyme ^{35,37}. Gels are stained with ethidium bromide, destained appropriately,
4 and an electronic image is obtained using usually a CCD camera. Electronic images can be then
5 processed using a densitometric software and a densitogram is obtained for each gel lane. A
6 DNA dispersion curve relating DNA length to electrophoretic mobility, based on all length
7 standards is determined from an analytical mobility function. From the profiles of irradiated and
8 unirradiated (or enzyme and untreated DNA populations), the number average length, \bar{L} , of
9 each DNA distribution is calculated and the DSBs or clusters will be calculated as described in
10 (Fig. 1) ³⁵.

11 In addition to the above approach using repair enzymes as damage probes, Georgakilas *et*
12 *al.* ^{30,33} have used polyamines (putrescine) for the detection of very closely spaced abasic (AP)
13 sites (1-5 bp apart), which are poorly detected by Nfo AP endonuclease. Based on the same
14 principle of enzyme-detection of clusters as enzyme sensitive sites, other groups have also used
15 neutral agarose gel electrophoresis and fraction of activity released (FAR) assay, hybridization
16 assay or plasmid nicking assay ^{38,39}.

17 Finally, in an attempt of a second independent method different groups have developed a
18 modified version of the neutral single cell gel electrophoresis (Comet assay) using again repair
19 enzymes as damage probes ^{19,40}. Since its early introduction in 1984 ⁴¹) and later in its alkaline
20 version in 1988 ⁴²), the single-cell gel electrophoresis (SCGE) or Comet assay a modified version
21 of the microgel electrophoresis, has been widely used for the detection of low levels of various
22 types of DNA lesions including SSBs, DSBs and oxidized bases as reviewed in ^{43,44}). The Comet
23 or microgel assay under neutral running conditions has been used for the detection of DSBs in a

1 variety of cells including lymphocytes ^{45,48}). To our knowledge there are very limited data on the
2 use of the neutral Comet ^{40,47}) or microgel assay ^{15,19}) for the detection of non-DSB clustered
3 DNA lesions using different repair enzymes as enzymatic probes. In all cases, incomplete
4 cleavage of lesions by the repair enzymes can lead to a detection of only a fraction of the clusters
5 (Fig. 1). This issue becomes very important especially in the case of high-LET radiations where
6 the density of the lesions is expected to be very high ⁴⁹). The clusters described in Fig. 1 are an
7 idealized form a simple cluster. In reality and particularly in the case of high-LET, one would
8 expect a complex DNA lesion, for example a DSB with 5-10 additional surrounding lesions ²⁰).
9 Therefore each cluster detected by the enzyme is only a fraction of the lesions participating in the
10 cluster.

11

1 **Table 1.** The different *E-coli* or human repair enzymes and their substrates used in the detection of
 2 oxidative clustered DNA lesions ^{7,50}.
 3

Repair enzymes used as damage probes	Substrates
<i>E. coli</i> Nfo protein or human hAPE1 (Endonuclease IV) Associated lyase activity	<u>Abasic</u> : Several types of abasic sites including oxidized abasic sites, abasic sites modified with alkoxyamines and DNA containing urea residues
<i>E.coli</i> Fpg protein or hOGG1 protein (DNA glycosylase) Associated lyase activity	<u>Oxypurines</u> : FapyAdenine, FapyGuanine, C8-oxoGuanine, some abasic sites, C8-oxoAdenine and to a lesser extent, other modified purines.
<i>E.coli</i> or human Nth1 protein (Endonuclease III) Associated lyase activity	<u>Oxypyrimidines</u> : Thymine residues damaged by ring saturation, fragmentation, or ring contraction including thymine glycol and uracil residues.

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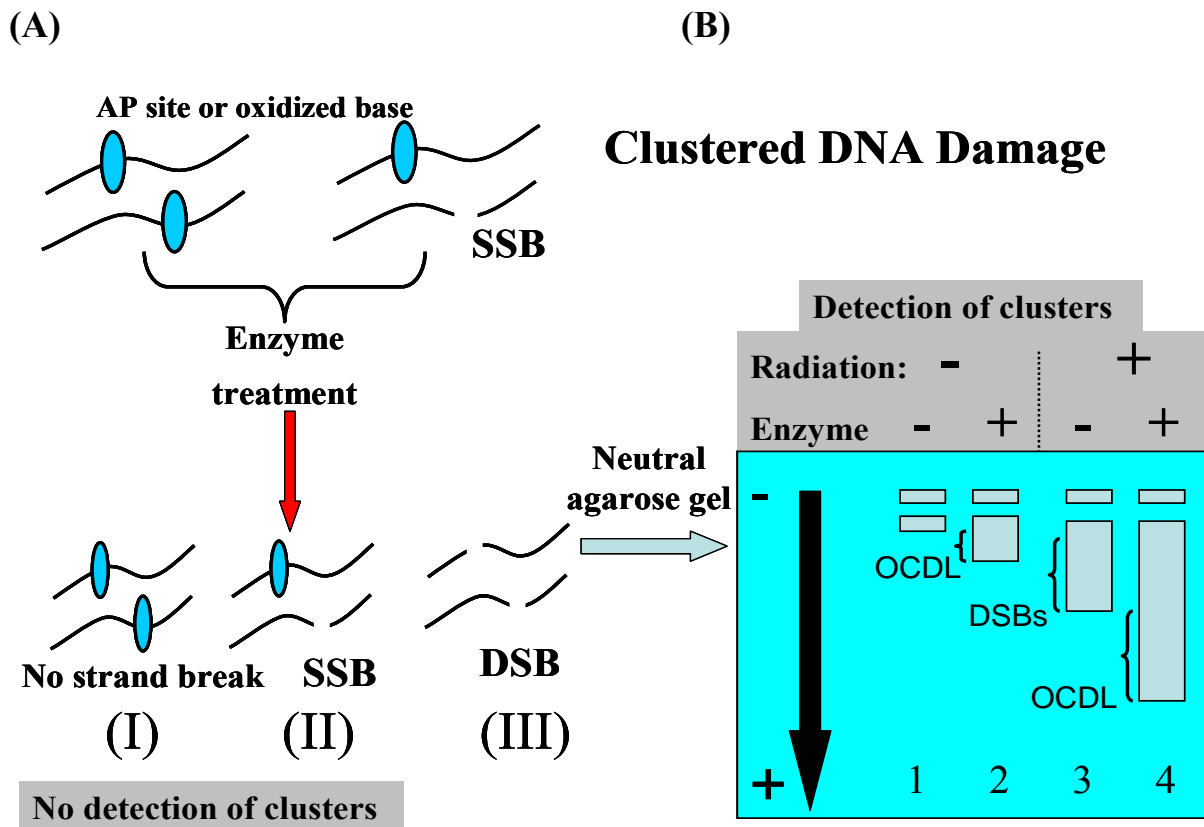


Fig. 1 Detection of bistranded clustered DNA lesions (oxidative clustered DNA lesions, OCDL) using neutral agarose gel electrophoresis. **(A)** Principles of detection using a repair enzyme for two representative type of clusters consisting of a set of bistranded base lesions or a base lesion and a single strand break, SSB. As shown in pathways I and II, incomplete cleavage of both lesions by the repair enzyme will lead to no detection of the cluster. In the case of cleavage of both lesions by the enzyme (pathway III) and induction of a DSB, detection of the cluster occurs. **(B)** Detection of clusters using neutral agarose gel electrophoresis. Genomic DNA (T7 or λ) or human DNA in agarose plugs can be subjected to agarose gel electrophoresis (constant or pulsed field according to the DNA size) and with application of number average length analysis (NALA) the prompt DSBs and OCDL can be measured in the same gel. Comparison of lanes 1 and 2 will provide the endogenous (non-irradiated) OCDL yields which usually are expected to be low. Comparison of lanes 3 and 4 provides the OCDL yields for the irradiated samples. In this case much higher levels of OCDL are expected. Comparison of lanes 3 and 1 will provide the yield of prompt DSBs induced directly by radiation. In every case the treatment with the enzyme results in an additional fragmentation i.e., DSBs which are equal to the number of clusters.

1 **INDUCTION OF CLUSTERED DNA LESIONS BY HIGH-LET RADIATIONS**

2 Theoretical considerations suggest that, in addition to isolated lesions, low-LET radiation can
3 create clusters with as many as 10 lesions ⁵¹⁾. High-LET radiation is capable of producing
4 damage of even greater complexity, i.e., up to 25 lesions per cluster ⁵¹⁾. Space travel
5 encompasses exposure to a broad spectrum of radiation ranging from the infrared to galactic
6 cosmic rays. The major component of galactic cosmic rays is the highly charged, energetic
7 (HZE) particles ranging from energetic protons to iron nuclei with energies ranging upwards to 1
8 GeV/nucleon; although of lower fluence than protons, large contributions to dose arise from Fe
9 and other very high LET nuclei, such as N, Si, etc. ⁵²⁾. Estimates of space radiation health risk
10 and the development of efficient countermeasures are key issues for manned space exploration.
11 Accurate risk calculations require a detailed investigation of both the physical aspects (patterns
12 of energy deposition at the molecular/cellular level) and the biological response to high LET
13 particles. The uncertainties in radiation risk assessment for deep-space missions are between 400
14 and 1,500% ⁵³⁾. These uncertainties are largely due to the lack of information on the biologic
15 response to HZE particles relative to the more extensively studied biological response to low
16 LET radiation. Late effects of high-LET radiation are arguably the health risk not only for the
17 human space exploration but also for increasing number of cancer patients treated by heavy-ion
18 therapy including young adults and children.

19 Measurement of DSBs for high LET radiations has been controversial ⁵⁴⁾. In addition, very
20 limited data exists for the efficacy of high-LET particles to induce OCDL in human cells
21 ^{10,34,55,56)}. Many early studies on DSB induction by radiation of different qualities have shown an
22 increased yield of DSBs with increasing LET in non-cellular systems ⁵⁷⁾ while other later studies
23 have shown a decline of DSBs with increasing LET for SV40 DNA under high radio-quenching

1 conditions ⁵⁸⁾ or plasmid DNA ³⁹⁾ irradiated in radio-quenching conditions mimicking the cellular
2 chemical environment. For mammalian cells though, relative biological effectiveness (RBE)
3 values for DSB induction have been usually found close or a little bit higher to unity ^{59,60)}. The
4 exclusion of smaller DNA fragments (<10 kbp) during PFGE analysis of DSBs can have a
5 significant impact on the measurement of DSBs and OCDL for high-LET radiations ⁶¹⁾.
6 Friedland *et al.* ⁶¹⁾ using the PARTRAC Monte Carlo simulation code have shown that the DSB
7 yield can drop significantly i.e., RBE values smaller than unity if during DSB measurements
8 DNA fragments smaller than 10 kbp are excluded. Experimental studies have shown the
9 importance of small DNA fragments (<200 kbp) especially in the case of high-LET charged
10 particles ^{62,65)} due to the non-random distribution of breaks ^{63,65)}. The advantage of using NALA,
11 especially in the case of high-LET radiations, is that this method does not require any specific
12 knowledge of the distribution of DNA fragments (e.g., random or Poisson-distributed cleavage)
13 ³⁷⁾. Another significant parameter related to the detection of clustered DNA lesions are the heat-
14 labeled sites within locally multiply damaged sites which are produced by radiation and are
15 subsequently transformed into DSBs during the lysis procedure. These artifactual DSBs can
16 consist 30-50% of the real DSBs induced by radiation depending on the lysis conditions (37°C or
17 50°C) ^{14,66)}.

18 In the case of non-DSB clusters the situation is clearer. A decrease has been detected for
19 OCDL yields and for high-LET radiations compared to low LET and RBE values much lower
20 than unity have been found by different groups using different DNA systems and methods
21 ^{34,56,67,68)}. Theoretical analysis and specifically Monte Carlo simulations predict that the initial
22 yield of clusters other than the DSB tends to decrease with increasing particle LET, which is
23 consistent with experimental observations ⁶⁹⁾. In addition, several *in vitro* studies using different

1 DNAs in radio-quenching solutions indicate a similar decrease of clusters with increasing LET
2 summarized also in Table 2^{34,68,70,71)}. This decrease could be the result of an actual decline in
3 the induced oxybase lesions⁷²⁾ and SSBs^{39,73)} for high LET charged particles associated with
4 decreased formation of radicals in the solution by ions of higher LET⁷⁴⁾, or an inability of the
5 methods to detect these highly dense clusters. By the last comment, we refer to the already
6 established compromised ability of repair enzymes to detect and cleave very closely spaced
7 DNA lesions^{29,33,75)}. Earlier studies using enzymatic (Fpg and/or Nth) or cell extract treatment
8 of γ - or α -irradiated plasmid DNA under cell mimetic conditions suggest an increase of the
9 contribution of base lesions to clustered DNA damage with LET⁷⁶⁾. Finally and related to all the
10 gel electrophoresis based methods for detection of clusters, an important issue that should be
11 mentioned here is that even if the enzymes cleave the resulting DNA fragments (<1 kbp) will
12 remain undetected under the most current electrophoresis separation regimens applied as
13 discussed above. Two general trends can be mentioned: i) For DSBs, a small dependence to
14 LET with a tendency to increase at least for LET values 1-150 keV/ μ m, for non-DSB clusters a
15 decline with LET and a linear dependence on dose for both DSBs and OCDL and ii) In most
16 cases, non-DSB cluster yields (Fpg-, EndoIII- and EndoIV-clusters) tend to be lower than the
17 corresponding yields of prompt DSBs for high-LET radiations. For low-LET radiations, the
18 non-DSB clusters tend to be higher compared to prompt DSBs (Table 2).

1 **Table 2.** Data available on the dependence of non-DSB clustered DNA damage to LET. The yields of
 2 DSBs and non-DSB clusters have been included. The different types of clusters are presented according
 3 to the type of repair enzyme used for the detection i.e., Fpg-, EndoIII and EndoIV-clusters. Yields are
 4 presented as lesions/Gbp/Gy.

5

LET (keV/μm)	Radiation	DSBs /Gbp/Gy	Fpg- /Gbp/Gy	EndoIII- /Gbp/Gy	EndoIV- /Gbp/Gy	Model System
1	⁶⁰ Co γ-rays	15		48		Plasmid pMSG-CAT
110	²³⁸ Pu α-particles	25		15		10 mM Tris Ref. ⁷¹⁾
1	⁶⁰ Co γ-rays	7.1		29.9		Plasmid pMSG-CAT
110	²³⁸ Pu α-particles	15		5		200 mM Tris Ref. ⁷¹⁾
0.3	¹³⁷ Cs γ-rays	0.81 x 10 ³		2.35 x 10 ³		Plasmid pEC
97	²⁴⁴ Cm α-particles	0.71 x 10 ³				10 mM phosphate buffer
145	⁵⁶ Fe	0.31 x 10 ³		0.35 x 10 ³		Ref. ⁷⁷⁾
1440	¹⁹⁷ Au	0.25 x 10 ³				
0.225	¹ H	9.56 x 10 ³	6.67 x 10 ³		4.09 x 10 ³	T7 DNA in PBS
0.3	¹³⁷ Cs γ-rays	3.78 x 10 ³				Ref. ⁶⁸⁾
2	X-ray	5.62 x 10 ³	10.05 x 10 ³		5.34 x 10 ³	
12.97	¹² C	3.72 x 10 ³	4.37 x 10 ³		2.04 x 10 ³	
50.32	²⁸ Si	3.57 x 10 ³	2.85 x 10 ³		1.27 x 10 ³	
107.7	⁴⁸ Ti	2.81 x 10 ³	2.42 x 10 ³		1.30 x 10 ³	
150.4	⁵⁶ Fe	1.95 x 10 ³	2.35 x 10 ³		1.22 x 10 ³	
0.3	¹³⁷ Cs γ-rays	11.9	10.68	11.87	9.5	Human monocytes
148	⁵⁶ Fe	10.9	7.12	8.54	5.5	Ref. ³⁴⁾

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1 Hada and Sutherland ⁶⁸⁾ and Tsao *et al.* ³⁴⁾ have also shown a prevalence of Fpg-clusters to
2 Endo IV (Nfo)-or EndoIII-clusters for T7 or human DNA irradiated with ⁵⁶Fe ions under radio-
3 quenching conditions (Table 2). As mentioned earlier, another effect that has to be taken into
4 consideration is the conversion of heat labile sites to strand breaks ⁷⁸⁾. It is a possible source of
5 overestimation of DSBs and non-DSB clusters and, especially in the case of γ rays, the
6 artifactual conversion of heat labile sites into DSB can result in the underestimation of RBE
7 values for Fe ions ⁵⁹⁾. In general, and as also stated by Boucher *et al.* ⁷⁹⁾, the role of artifactual
8 DNA oxidation is very important but based on the above different group data, its role in the case
9 of high-LET radiations is expected to be minimum. Finally as shown in Table 1, the overlapping
10 specificity of the different repair enzymes is another potential source of overestimation in the
11 measurement of total number of non-DSB clusters that can reach in some cases even a factor of
12 two ⁴⁰⁾.

13

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CONCLUDING COMMENTS

15 Although a significant amount of data has been produced on the efficiency of high-LET
16 radiations to induce clustered DNA damage, still many questions remain unanswered.
17 Significant deviations appear between theoretical and experimental data. Therefore, we believe
18 that the primary objectives of the future studies should be to i) provide analytical, quantitative,
19 and theoretical information on the induction and processing of different patterns of complex
20 DNA damage (DSB and non-DSB clustered DNA lesions) produced directly and indirectly
21 (bystander effects) by high-LET radiations *in vivo* and *in vitro* and ii) measure the biological
22 effectiveness of high-LET radiations (like space HZE particles or α -particles) for the induction of
23 chromosome damage and apoptosis *in vivo* and *in vitro* and correlate these endpoints with the

1 levels of complex DNA damage detected in each case, and finally iii) improve the experimental
2 detection methods for including the very small DNA fragments expected to be induced in the
3 case of high-LET radiations. A better knowledge of the induction and repair of complex DNA
4 damage is essential for the calculations of the risk factors of high LET radiations to induce
5 carcinogenesis (as in the case of radon-gas emitted α -particles, heavy-ion therapy or space
6 radiation).

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